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The overall goal of this research project is to explore the roles of TGF-ß and components of its signaling pathways in the initiation, progression and metastasis of breast adenocarcinomas through an investigation of the disregulation of TGF-ß signal transduction. Last year, we identified and isolated three cDNAs encoding members of the Smad family and studied the TGF-ß induced phosphorylation of these molecules in a normal mammary epithelial cell line. Subsequently, we have focused on the functional role of Smad3 and Smad4 as tumor suppressors in mediating the TGF-ß signal in transactivating downstream target genes. We have extended our analysis of the biological activity of the Smad proteins in TGF-ß signaling by studying the nuclear activity of Smad2, Smad3 and Smad4. Results from further analysis in these directions will not only significantly contribute to an understanding of the molecular events leading to breast carcinogenesis, but also aid in the development of new therapeutics for breast cancer.

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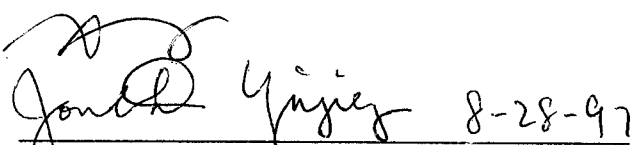

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INTRODUCTION

Breast cancer is the most common cancer in women in the United States. Although endocrine therapy has proven useful in treating approximately one-third of these patients, the tumors inevitably progress to a hormone independent state and no longer respond to these therapies. Thus, it is the goal of this research project to characterize the molecular basis of the TGF-β growth inhibitory in hopes of elucidating additional therapeutic targets.

TGF-βs are a group of multifunctional peptide hormones that play critical roles in many normal cellular processes including the regulation of proliferation, differentiation, extracellular matrix deposition, cell adhesion and migration (Yingling et al., 1995). Perhaps TGF–βs most critical biological activity is its ability to inhibit the growth of a wide variety of cell types including breast epithelia (Sporn and Roberts, 1985). Therefore, loss of components of the TGF–β growth inhibitory pathway in breast epithelia would be expected to contribute to the genesis and progression of breast cancer. Loss of TGF–β responsiveness in MCF-7 cells has been correlated with loss of the type II TGF–β receptor and increased tumorigenesis. Reintroduction of the TGF–β type II receptor in these MCF-7 cells reverses this tumorigenic phenotype indicating the importance of this pathway in preventing breast tumorigenesis (Sun et al., 1994).

Significant progress has been made over the last 5 years to elucidate the molecular events at the cell membrane involving receptor activation which initiate TGF–βs intracellular signaling pathway. In addition, nuclear events involving induction of cyclin-dependent kinase inhibitors (p15 and p21) have been implicated in mediating TGF–βs growth inhibitory signal (Li et al., 1995), (Datto et al., 1995). However, the nature of the cytoplasmic signaling cascade which transduces the signal from the membrane to the nucleus is just beginning to be resolved. Genetic analyses in *Drosophila* and *C. elegans* have led to the isolation of a variety of components of the TGF–β-like pathway including ligands and receptors which are homologous to those identified in vertebrates. The most recent addition to the list of genetically implicated proteins in *Drosophila* and *C. elegans* is the MAD (*Drosophila*) or SMA (*C. elegans*) family of proteins (Sekelsky et al., 1995),

(Savage et al., 1996). Mutation in these genes causes phenotypes analogous to those of the TGF–β-like receptors in these organisms thus implicating them in a downstream signaling pathway. Additional evidence from vertebrates is the discovery of a tumor suppressor gene on chromosome 18q which is deleted in ~50% of human pancreatic tumors (Hahn et al., 1996). This gene, called DPC4, is a member of the MAD and SMA family by virtue of it containing the invariant MH1 and MH2 domains at its amino- and carboxy-termini, respectively.

The molecular basis of the role of the Smad family in the TGF–β pathway has begun to be elucidated. Our previous work on the phosphorylation of endogenous Smad proteins in response to TGF-β and BMP (Yingling et al., 1996) has been verified by numerous groups using *in vitro* phosphorylation assays. Taken together these data suggest that Smad2 and Smad3 are inducibly phosphorylated in response to TGF-β and that Smad1 and Smad5 are phosphorylated in response to BMP signals. An association between Smad1,2,3 or 5 and Smad4 correlates with their phosphorylation (JMY, unpublished data; and Lagna et al., 1996) and their nuclear accumulation. We have extended our analysis of the biological activity of the Smad proteins in TGF-β signaling by studying the nuclear activity of Smad2, Smad3 and Smad4. The results from these studies are summarized in the next section and a copy of a submitted manuscript is included in the Appendix.

PROGRESS REPORT

Members of the Smad family of proteins are thought to play important roles in transforming growth factor-β mediated signal transduction (Attisano and Wrana, 1996; Massague, 1996). In response to TGF-β, specific Smads become inducibly phosphorylated (Eppert et al., 1996; Lechleider et al., 1996; Yingling et al., 1996), form heteromers with Smad4 (Lagna et al., 1996), and undergo nuclear accumulation (Baker and Harland, 1996; Hoodless et al., 1996; Liu et al., 1996). In addition, over-expression of specific Smad combinations can mimic the transcriptional effect of TGF-β on both the PAI-1 promoter as well as the reporter construct, p3TP-Lux (Lagna et al., 1996; Liu et al., 1996; Macias-Silva et al., 1996; Zhang et al., 1996). Although this data suggests a role for Smads in regulating transcription, the precise nuclear function of these heteromeric Smad complexes remained unknown. Therefore, my work over the past twelve months has focused on the nuclear activity of the Smad proteins.

We have shown that in Mv1Lu cells Smad3 and Smad4 form a TGF-β induced, phosphorylation dependent, DNA-binding complex that specifically recognizes a bipartite binding site in p3TP-Lux. Furthermore, we demonstrated that Smad4 itself is a DNA binding protein which recognizes this same sequence. Interestingly, mutations which eliminate the Smad DNA binding site do not interfere with either TGF-dependent transcriptional activation or activation by Smad3/Smad4 co-overexpression. In contrast, mutation of adjacent AP1 sites within this context eliminates both TGF-β-dependent transcriptional activation and activation in response to Smad3/Smad4 co-overexpression. Taken together, this data suggests that the Smad3/Smad4 complex has at least two separable nuclear functions: it forms a rapid, yet transient sequence-specific DNA-binding complex and it potentiates AP1-dependent transcriptional activation.

My work over the past three years which has been supported by the DOD Army Breast Cancer Initiative has significantly contributed to our current understanding of the biological activity of the Smad family of proteins which are involved in TGF-β superfamily signaling pathways. Although there are many unresolved issues with regard to the precise molecular mechanism utilized by these proteins, the basic fundamentals of their biological function have been elucidated. The Smads are a highly conserved family of intracellular

signaling proteins which form heteromers and translocate to the nucleus in response to inducible phosphorylation mediated by specific TGF-β superfamily signaling pathways. Once in the nucleus they have the capacity to directly bind DNA and to affect transcription of specific genes independent of their DNA-binding activity. Thus, these novel proteins are multifunctional transcriptional regulators and represent the serine/threonine kinase receptors equivalent of the STAT proteins for receptor tyrosine kinases.

CONCLUSIONS

Significant progress has been made in advancing the goal described in the Specific Aim 2 of the original proposal. As discussed in the section of Introduction, available evidence strongly suggest that Smads play a more global role as regulators of multiple biological responses to ligands of the TGF-β superfamily by participating in transcriptional activation of multiple target genes. Specifically for TGF-β, Smads may act as intermediates for a number of TGF-β signaling pathways leading to cell growth regulation and extracellular matrix deposition. Loss of function mutations in the Smads could disrupt those important TGF-β signaling pathways and contribute to tumorigenesis in multiple types of tissues and organs, including the breast, in humans. Inside the nucleus, Smads may work to modulate transcription by binding to specific DNA elements in promoters of the target genes and interacting with a spectrum of different DNA-binding transcription factors or factors of the basal transcription machinery. As described in the last section, our recent progress in determining the biochemical properties of Smads in mediating the biological effects of TGF-β, with the collagenase and PAI-1 gene promoters as model systems, has provided us with an opportunity for studying this novel TGF-β signaling mechanism, a key step toward an understanding of how this multifunctional hormone regulates so many cellular functions. A comprehensive analysis of the role of Smads in modulating the expression of endogenous PAI-1 gene, combined with a determination of the identities and physiological roles of additional genes which are potentially under the control of this signaling pathway, will lead to further elucidation of the program of TGF-β signal transduction. The exploration of the physiological role of Smad3 by the strategy of genetic manipulation of the Smad3 gene will address the question whether Smad3, like Smad2 and Smad4, function as a tumor suppressor in animals.

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APPENDIX

1. Yingling, J. M., Datto, M. B., Wong, C., Frederick, J. P., Liberati, N. T., Wang, X.- F. (1997) The tumor suppressor, Smad4, is a TGF-β -inducible DNA binding protein. Submitted.

The Tumor Suppressor, Smad4, is a TGF-β-Inducible DNA Binding Protein

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Abstract

Members of the Smad family of proteins are thought to play important roles in transforming growth factor-β mediated signal transduction. In response to TGF-β, specific Smads become inducibly phosphorylated, form heteromers with Smad4, and undergo nuclear accumulation. In addition, over-expression of specific Smad combinations can mimic the transcriptional effect of TGF-β on both the PAI-1 promoter as well as the reporter construct, p3TP-Lux. Although this data suggests a role for Smads in regulating transcription, the precise nuclear function of these heteromeric Smad complexes remains unknown. Here we show that in Mv1Lu cells Smad3 and Smad4 form a TGF-β induced, phosphorylation dependent, DNA-binding complex that specifically recognizes a bipartite binding site within p3TP-Lux. Furthermore, we demonstrate that Smad4 itself is a DNA binding protein which recognizes this same sequence. Interestingly, mutations which eliminate the Smad DNA binding site do not interfere with either TGF-dependent transcriptional activation or activation by Smad3/Smad4 co-overexpression. In contrast, mutation of adjacent AP1 sites within this context eliminates both TGF-β-dependent transcriptional activation and activation in response to Smad3/Smad4 co-overexpression. Taken together, this data suggests that the Smad3/Smad4 complex has at least two separable nuclear functions: it forms a rapid, yet transient sequence-specific DNA-binding complex and it potentiates AP1-dependent transcriptional activation.

Introduction

Transforming growth factor-β (TGF-β) is a multipotent peptide hormone which regulates a diverse array of biological processes (21). The involvement of TGF-β in the pathogenesis of several diseases has resulted in intense investigation of its molecular mechanism of signal transduction (22). Several years ago the signaling receptors for TGF-β were cloned and found to be transmembrane serine/threonine kinases termed the type I and type II receptors (3, 17). Although the molecular nature and mechanism of activation for these TGF-β receptors at the cell surface has been described (28, 29), the intracellular pathways which transduce the TGF-β signal from the membrane to the nucleus has only recently begun to be elucidated.

Genetic studies in *Drosophila* (24) and *C. elegans* (23) identified a conserved family of proteins as playing a critical role in TGF-β superfamily signaling pathways downstream of the receptors. Mammalian homologs of these proteins, now referred to as Smads (8), were subsequently cloned and characterized (1, 20). Studies in *Xenopus* embryos has revealed a functional division between the mammalian Smad proteins. Smad1 (10, 18, 27) and Smad5 (25) have been shown to induce ventral mesoderm and thus mediates the BMP signal, while Smad2 transduces TGF-β signals and induces dorsal mesoderm (2, 10). The distantly related Smad4 protein which was originally identified as a tumor suppressor protein on chromosome 18q (11), induces both ventral and dorsal mesoderm and thus mimics TGF-β and BMP signals (33). Smad4 has been shown to associate with Smad1 in response to BMP and with Smad2 in response to TGF-β and thus is a common component of these signal transduction pathways (15). The Smads have been found to be inducibly phosphorylated in response to TGF-β and BMP and the ligand-specific nature of the Smads have been confirmed by these studies. Smad2 and Smad3 are specifically phosphorylated in response to TGF-β (9, 16, 31, 32), while Smad1 is phosphorylated

in response to BMP (12, 31). Phosphorylation of the Smads results in their heteromerization with Smad4 (15), and correlates with their accumulation in the nucleus (2, 12, 18). Recently, the type I receptor was found to be the kinase responsible for ligand inducible phosphorylation of C-terminal serine residues of Smad1 in response to BMP (14) and Smad2 in response to TGF-β (19). The C-terminal domain of Smad1 and Smad4 has been shown to possess transcriptional activation activity in the context of a Gal4-DNA-binding domain fusion (18), thus providing the first indication of a nuclear function for the Smad proteins. Subsequently, over-expression of specific Smad combinations has been found to mimic the transcriptional effect of TGF-β on both the PAI-1 promoter as well as the reporter construct, p3TP-Lux (5, 15, 18, 19, 32). Smad4 has been shown to be required for this transcriptional activity since Smad4-deficient cell lines are non-responsive, but can be rescued with Smad4 expression (7, 15). Interestingly, the homomeric and heteromeric interactions between Smad3 and Smad4 correlate with their ability to transcriptionally activate the PAI-1 reporter (30). Furthermore, naturally occurring Smad4 mutations interfere with its ability to associate with Smad3 (30). Although these data suggest a role for Smads in regulating transcription, the precise nuclear function of the heteromeric Smad complexes remains unknown.

Here we demonstrate that Smad3 and Smad4 participate in a DNA-binding complex on a fragment of the p3TP-Lux reporter and that Smad4 is the DNA-binding component of this complex. In the context of this reporter, the Smad binding site is not required for transcriptional activation in response to TGF-β nor Smad3/Smad4 co-overexpression. However, we also show that an endogenous promoter, the plasminogen activator inhibitor-1 (PAI-1) promoter, contains a Smad binding site. Thus, the ability of Smad3/Smad4 to directly bind DNA may have physiological relevance in regulating transcription of TGF-β responsive genes.

Materials and Methods

Cell Culture. Mink lung epithelial cells (Mv1Lu) were obtained from ATCC and maintained in DMEM with 10% FBS, penicillin and streptomycin and non-essential amino acids. COS cells were maintained in DMEM with 10% FBS, penicillin and streptomycin.

Plasmid Construction. Flag-tagged human Smad4, human Smad3 and human Smad2 were the generous gift of Dr. Rik Derynck. Expression vectors for Smad3 WT and Smad3MT (3S‡A) were generated by PCR using the following primers: Smad3 WT; 5' primer: GGATCCGCGATGTCGTCCATCCTGCCTTTCAC and 3' primer: GGATCCTAAGACACACTGGAACAGC; Smad3MT (3S‡A); 5' primer: same as Smad3 WT above and 3' primer: GGATCCTAAGCCACAGCTGCACAGCGGATGCTTGG. The resulting BamHI fragments were cloned in-frame with the HA tag in pCGN (26). p3TP-Lux (28) and pGL2-T+I have been previously described (6). The luciferase reporter constructs 4X WT, 4X SBS Dbl mutant and 4X AP1 Dbl mutant were created using the following oligos: 4X WT; GGATGAGTCAGACACCTCTGGCTGTCCGGAAG and TCCCTTCCGGACAGCCAGAGGTGTCTGACTCA, 4X AP1 Dbl mutant; GGATACAGCAGACACCTCTGGCTGTCCGGAAG and TCCCTTCCGGACAGCCAGAGGTGTCTGCTGTA, 4X SBS Dbl mutant; GGATGAGTCACTGCATTCTGGCTGTCCGGAAG and TCCCTTCCGGACAGCCAGAATGCAGTGACTCA. 2X directional constructs were created by first phosphorylating the above oligo sets, annealing and ligating in the presence of 0.5x molar ratio of phosphorylated and annealed linker oligos: short linker, GGCTCGAGAGATCT; long linker 5': TCCAGATCTCTCGAGCC and long linker 3': GGAAGATCTCTCGAGCC. The resulting ligation was digested with BglII and cloned into the BglII site of pGL2 T+I. Constructs which contained two inserts in a backwards orientation where then cut with XhoI and EcoRV. The two site containing fragments were then cloned into the XhoI and SmaI sites in reporter constructs which contained two inserts in a forwards orientation to produce constructs with four inserts in the

same orientation. GST-Smad3 and GST-Smad4 were created by PCR from plasmid templates using the following primers: Smad3; 5'primer:

CGGGATCCCGATGTCGTCCATCCTGCCTTTCAC, 3'primer : same as Smad3 WT above; Smad4; 5'primer: CGGGATCCCGATGGACAATATGTCTATTACG, 3' primer:

GGATCCTCAGTCTAAAGGTTGTGGG. The resulting BamHI fragments were cloned in-frame into pGex3X-HMK (Pharmacia).

Electrophoretic Mobility Shift Assays. Extracts were prepared from approximately $2x10^6$ COS cells transiently transfected with Smad expression constructs using a standard DEAE-Dextran transfection protocol (28) or from approximately $2x10^6$ Mv1Lu control cells or cells treated with 100 pM TGF-β for 30 min after 2 hours of serum starvation. Cells were then lysed and nuclear extracts were prepared as previously described (6). Whole cell extracts prepared in a lysis buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% NP40, 50 mM NaF, 1 mM DTT, 1 mM PMSF, 1mM sodium orthovanidate, and protease inhibitors, gave identical gel shift results. For phosphatase treatment, 2 U of CIAP (Boehringer Mannheim) and 0.06 U of PAP (Boehringer Mannheim) were added to 100 ul of nuclear extract (prepared without phosphatase inhibitors) and incubated at 37°C for 30 min in the presence or absence of phosphatase inhibitors; 1mM sodium orthovanidate, 10 mM NaF, 10 mM β-glycerophosphate, and 0.2 mM sodium molybdate. α^{32}P-dTTP Klenow labeled oligos used for probes were as shown in Fig. 2A. Alternatively wild type probe was created by digesting p3TP-Lux with SphI and NdeI and α^{32}P-dTTP Klenow labeling. The PAI-1 promoter probe was obtained by restriction digest with NcoI and EagI and α^{32}P-dTTP Klenow labeling. Gel shift condition were as follows: 1.5 uL of nuclear extract (or 3 ul whole cell extract) containing approximately 3 ug of protein, 1 ug of dIdC and 0.5 ng of probe labeled to an activity of 10,000 - 40,000 cpm/0.5 ng were brought to a final volume of 15 ul using a hypotonic lysis buffer as previously described (6). For supershift analysis a rabbit polyclonal Smad4

antibody was created against full length GST-Smad4 by standard protocols. Pre-immune serum is from the same rabbit. Anti-HA antibody was obtained from Boehringer-Mannheim, the Pan Fos antibody was obtained from Santa Cruz (K-25) and anti-Flag antibody (M2) was obtained from Kodak IBI. 2 ul of each antibody was used for supershifts. For gel shifts with eluted GST-Smad3 and Smad4, approximately 100 ng of protein in 1 ul of a buffer containing 100mM Tris pH 8, 120 mM NaCl, 25 uM glutathione was used. Complexes were resolved on a 6% acrylamide, 0.04% bis-acrylamide, 0.5X TBE gel as previously described (6), except for the HA supershift panel of Figure 2B which was resolved on a 6% acrylamide, 0.2% bis-acrylamide, 0.5X TBE gel.

Methylation Interference. Methylation interference probes were prepared as above with the following exceptions: 4 ul of DMS was added to 100 ul of the Klenow labeling reaction which contained 1 ug total of DNA. After a 5 min room temperature incubation 40 ul of 1.5M sodium acetate and 1M β-mercaptoethanol was added. Probe was then precipitated with the addition of 0.5 ml of 100% ETOH. Probe was then gel purified and used in EMSA as described above. After a short -80°C exposure of the unfixed/undried EMSA gel, shifted complexes were cut out and bound probe was electro-eluted, precipitated and resuspended in 100 ul of 1M piperidine. Samples were then heated to 90°C for 30 min, and piperidine was subsequently removed by several rounds of lyophilization and resuspension in distilled water. The resulting cleaved products were resolved on a urea/acrylamide sequencing gel.

Luciferase Assays. Transfections were performed using a standard DEAE-Dextran transfection protocol (28). Luciferase assays were performed as previously described (6). All transfections are normalized to β-galactosidase activity by co-transfection of 0.5 ug of a CMV-β-gal expression vector . Quantities of DNA transfected are detailed in the figure legends.

Western Blot Analysis. Proteins from COS transfected lysates were resolved by 8% SDS-PAGE and transferred to Immobilon (Millipore). The blots were blocked in B/P solution (50 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween-20) containing 2% milk. Primary antibody (HA, Flag or Smad4) was added in B/P solution at 1:1000 for 1 hr at room temperature. The blots were washed 3X with B/P solution and the appropriate secondary antibody (Bio-Rad) was added (goat anti-mouse for HA/Flag and goat anti-rabbit for Smad4) for 1 hr at room temperature. After washing 3X with B/P solution, the blots were developed with ECL (Amersham) and exposed on Kodak XAR5 film.

Results

Smad3/Smad4 Co-Overexpression Regulates Transcription. The p3TP-Lux luciferase reporter is a well described and widely used artificial promoter construct which was empirically designed to have maximal responsiveness to TGF-β (28). p3TP-Lux has a 31-nucleotide, AP1 site containing region of the collagenase promoter, concatamerized 5' to an ~400 nucleotide region of the PAI-1 promoter followed by 70 bp of the adenovirus E4 promoter (Fig. 1A). Consistent with previous findings, we observe a transcriptional activation of p3TP-Lux by both Smad3/Smad4 co-overexpression and TGF–β treatment in Mv1Lu cells (Fig. 1B). In contrast, Smad2 fails to activate transcription of p3TP-Lux when co-overexpressed with Smad4. To define the Smad responsive region of p3TP-Lux, we created a reporter construct comprised only of the 31-nucleotide AP1 site containing region concatamerized 5' to a minimal promoter. This 4X WT reporter (Fig. 1A) is not only TGF-β-responsive, but is also activated in response to Smad3/Smad4 co-overexpression (Fig. 1C). Thus, this 31-nucleotide repeat contains a DNA sequence which is both TGF-β and Smad responsive.

Smad3/Smad4 Participates in a DNA-Binding Complex. To determine if Smad3/Smad4 co-overexpression changes the DNA-binding complexes on this 31-nucleotide fragment, we performed gel shifts using a probe consisting of two copies of the 31-nucleotide repeat cut from p3TP-Lux, termed the 2.0 probe (Fig. 3A). When gel shifts were performed using this probe and extracts derived from COS cells co-transfected with epitope tagged Smad3 and Smad4 (Fig. 2A), we observed not only an AP1 containing complex (Complex I), but also a strong additional binding complex (Complex II, Lane 6). Overexpression of Smad3 alone produces a lower level of a complex with similar mobility (Lane 3). Likewise, overexpression of Smad4 produces a complex with similar mobility, as well as a slightly faster migrating complex (Lane 4). In contrast, Smad2/Smad4 co-expression does not produce this complex, but appears similar to Smad4 alone (Lane 5).

One possible explanation for these observations is that Smad3 and Smad4 form a DNA binding complex. Overexpressed Smad3 alone or Smad4 alone could bind DNA with their endogenous Smad partner, whereas co-overexpression would produce a large amount of Smad3/Smad4 binding complex. To test this hypothesis, supershift analysis was performed to determine if HA-tagged Smad3 or Flag-tagged Smad4 are present in the additional binding complex (Complex II). As shown in Figure 2B, both HA and Flag antibodies supershift this complex (Lanes 7, 12 and 13). As expected, a Pan-Fos family member antibody supershifts the faster migrating AP1 complex (Lane 2). This antibody, however, does not shift the Smad3/Smad4 complex (Lane 8), suggesting that although the constitutive binding activity contains a Fos family member, the Smad3/Smad4 complex does not. Finally, the complexes observed with Smad4 overexpression are all Smad4 containing as demonstrated by Flag supershifts (Lane 5). Thus, Smad3 and Smad4, when overexpressed, participate in a DNA binding complex on sequences present in this region of p3TP-Lux.

Recently, the BMP-inducible phosphorylation sites of Smad1 and the TGF-β-inducible phosphorylation sites of Smad2 have been identified (14, 19). Smad3 contains analogous sites of potential phosphorylation at its C-terminus. Based on this sequence homology, we created a

phosphorylation deficient mutant of Smad3, Smad3MT, and assayed its ability to participate with Smad4 in a DNA binding complex. Although the expression levels were similar to wild-type Smad3, Smad3MT was unable to form a DNA binding complex with Smad4 (Fig. 2A, Lane 7). The results with this mutant suggest that an intact carboxyl-terminus of Smad3 is essential for formation of the DNA binding complex. This mutation possibly interferes with the ability of Smad3 to form a heteromeric complex with Smad4 and thus precludes formation of the DNA-binding complex.

Isolation of the Smad DNA-Binding Element. To more precisely determine the DNA sequences to which the Smad3/Smad4-containing complex binds, we systematically mutated the 2.0 probe (Fig. 3A). As expected, mutation of the AP1 binding sites eliminated the Fos-containing shifted complex. The Smad3/Smad4 complex, however, was still present on the AP1 site mutant probe, although in somewhat decreased amounts (Fig. 3B, Lane 3). This further suggests that the Smad3/Smad4 complex is not binding through AP1. We next designed three separate scanning mutants to encompass the entire 2.0 probe in search of the specific sequence which confers Smad3/Smad4 binding (Fig. 3A). As shown in Figure 3B, scanning mutant #1 eliminates both the AP1 and the Smad3/Smad4 complexes, while scanning mutant #2 specifically eliminates the Smad3/Smad4 complex leaving the AP1 complex intact. Scanning mutant #3 has no effect on the binding of either complex. Thus, the region necessary for Smad3/Smad4 complex binding lies within the bases mutated in scanning mutants #1 and #2.

Methylation interference was used to more precisely define which guanine residues within the 2.0 probe are contacted by the Smad3/Smad4 complex. The results shown in Figure 3C confirm the mutagenesis results in that there is a single protected guanine residue that is located within the region predicted by the scanning mutagenesis. Both sites of this two site probe have almost completely protected guanine residues. This suggests that both sites are being contacted in this single Smad3/Smad4 complex. Mutation of 6 nucleotides surrounding this protected guanine (GACACC) in either the 5' or 3' site of the 2.0 probe was sufficient to eliminate Smad3/Smad4

binding (Fig. 3D), further indicating the requirement of a bipartite site for Smad3/Smad4 complex formation. In addition, a probe containing only one of these 31-nucleotide repeats (one half of the probe used in these experiments) was completely unable to bind the Smad3/Smad4 complex in gel shift assays (data not shown).

Smad4 Directly Binds DNA. Having demonstrated that overexpressed Smad3/Smad4 participates in a DNA-binding complex on the 2.0 probe, we next sought to determine if either Smad3 or Smad4 themselves were directly binding this DNA sequence. Therefore, we generated GST fusions of both proteins and used these purified reagents in gel shifts with the 2.0 probe (Fig. 4). Although Smad3 is incapable of binding (Lanes 1 and 2), GST-Smad4 directly binds the 2.0 wild type (Lanes 3 and 4) and AP1 mutant probes (Lane 6), but does not bind the 2.0 Smad binding site mutant probe (Lane 5). The DNA-binding protein was confirmed to be Smad4 by antibody supershift analysis. The Smad4 specific immune antisera alone produces a background DNA-binding band (Lane 7). The Smad4 antibody eliminates the 2 specific DNA-binding complexes (Lane 9), while the preimmune serum has no effect (Lane 10). Thus, the complex seen on Smad3/Smad4 co-overexpression may be the result of a direct DNA interaction by Smad4. Although Smad4 binds directly to this DNA sequence, Smad3 may modulate its binding affinity or affect its binding specificity. An altered specificity of Smad4 in complex with different Smads may explain why Smad2/Smad4 complexes do not bind this sequence, but Smad3/Smad4 complexes do. This altered specificity of Smad4 DNA-binding in complex with different Smads would be required to maintain the specific transcriptional events that occur in response to TGF-β superfamily ligands. The ability of Smad4 to directly bind DNA explains the additional shifted complex observed when Smad4 is overexpressed alone in COS cells (Fig. 2A and B, Lanes 4); it is Smad4 bound without endogenous Smad3. The ability of the Flag antibody to supershift this complex confirms the presence of Smad4 in this complex (Fig. 2B, Lane 5).

TGF-β Induces a Smad DNA-Binding Complex *In Vivo*. Mv1Lu cells are highly responsive to TGF-β and have been used as a model system to define various aspects of TGF-β mediated signal transduction. Thus, we used Mv1Lu cells as a model system to look *in vivo* for a Smad containing DNA-binding complex. Since the Smad proteins are known to be cytoplasmic proteins which translocate to the nucleus in response to ligand-induced phosphorylation, an endogenous Smad-containing DNA-binding complex would be predicted to be TGF-β-inducible and phosphorylation dependent. To examine this question, we performed gel shifts with the 2.0 probe and nuclear extracts prepared from either TGF-β treated or untreated Mv1Lu cells. In the absence of TGF-β treatment, Mv1Lu cells contain a constitutive Fos-containing binding complex similar to the Fos complex in COS cells (Fig. 5C, Lane 2). Upon TGF-β treatment, a slower migrating complex appears within 5 min, peaks in 15 min and disappears after 4 hrs. (Fig. 5A). This time course parallels the TGF-β-dependent phosphorylation kinetics of endogenous Smad proteins (31). In addition, the inducibly bound complex is sensitive to phosphatase treatment, suggesting that its binding is phosphorylation dependent (Fig. 5B). Thus this inducible complex has the characteristics expected for a Smad-containing DNA-binding complex. The presence of Smad4 in this TGF-β inducible complex was confirmed by the ability of a Smad4-specific antibody to eliminate formation of this complex (Fig. 5C, Lane 5).

Unfortunately, our Pan-Smad antibodies which recognize Smad1, 2, 3 and 5 (31) could not supershift either the endogenous Smad4-containing complex nor the Smad3/Smad4 co-overexpressed complex from COS cells because of their relatively low affinity for Smad3 (data not shown). Therefore, we cannot unequivocally show that Smad3 is a component of the TGF-β-inducible shifted complex in Mv1Lu cells. However, the inducible complex comigrates with the Smad3/Smad4 complex from COS lysates (data not shown) and shares an identical binding site

within the 2.0 probe as revealed by gel shift analysis using the panel of 2.0 probe mutants (Fig. 5D). These data combined with the fact that no other Smad in combination with Smad4 from COS lysates is able to bind the 2.0 probe, provides strong evidence that the inducible complex in Mv1Lu cells contains Smad3 and Smad4.

Functional Analysis of the Smad-DNA-Binding Element. Having identified the specific region of the 2.0 probe which was capable of conferring Smad3/Smad4 binding, we examined the functional consequences of Smad binding site and AP-1 site mutations in the context of the 4X WT reporter in Mv1Lu cells. As shown in Figure 6, the AP1 sites are critically important for induction by both TGF-β and Smad3/Smad4 co-overexpression. Surprisingly, mutation of the Smad binding site had no effect on induction by TGF-β or by Smad3/Smad4 co-overexpression. These results suggest that the heteromeric Smad3/Smad4 complex has at least two distinct nuclear activities. First, it rapidly forms a transient, sequence-specific DNA-binding complex with unknown function and secondly, it directly or indirectly potentiates AP1-dependent transcriptional regulation in the context of the p3TP-Lux reporter.

The Endogenous PAI-1 Promoter Contains a Smad DNA-Binding Element. The p3TP-Lux reporter is an artificial construct designed empirically for maximum TGF-β responsiveness. Although it has been instructive to biochemically define a novel binding function for Smad complexes, the question remains; does this Smad complex form on endogenous or native promoter sequences? To address this question, we examined endogenous promoters that are known to be activated by TGF-β and Smad co-overexpression for their ability to bind a Smad3/Smad4 containing complex. One such DNA sequence is an 800 bp stretch of the PAI-1 promoter. The TGF-β responsive region of the PAI-1 promoter has been described and surrounds a putative AP1 binding site (Fig. 7A and 13). The similarity of this endogenous sequence to that

created in p3TP-Lux made it an ideal candidate for study. Using a probe that encompasses this AP1 site, we discovered that indeed Smad3 and Smad4 co-overexpression in COS cells leads to an additional DNA-binding complex (Fig. 7B, Complex II). Thus, the ability of Smad3/Smad4 to bind DNA which we originally defined in the context of p3TP-Lux may have physiological relevance in TGF-β's ability to regulate endogenous promoters such as PAI-1. Studies are currently underway to investigate the functional role of this Smad-binding region within the PAI-1 promoter.

Discussion

An intracellular pathway for mediating TGF-β superfamily signals from the membrane to the nucleus has begun to be elucidated. This highly conserved pathway involves the Smad proteins, which have been shown to be phosphorylated by the type I receptor within the heteromeric signaling complex at the cell surface, to form heteromers with Smad4 and to accumulate in the nucleus. In this study, we have investigated the molecular nature of the Smads ability to transcriptionally activate the p3TP-Lux reporter.

Overexpression of Smad3 and Smad4 was found to activate transcription from p3TP-Lux in a ligand-independent fashion in Mv1Lu cells. Overexpression of Smad3/Smad4 results in the formation of a specific DNA-binding complex on the responsive region of the promoter. The inability of Smad2 and Smad4 to form a similar DNA-binding complex on this region correlates with their failure to activate transcription from the p3TP-Lux promoter. Thus, the heteromeric Smad4 complexes which are formed as a result of ligand-induced phosphorylation of Smad2 and Smad3 may have distinct nuclear targets. Recently, Smad2 has been found in a DNA binding complex with the transcription factor, FAST1 (4). Although the presence of Smad4 in this complex has not yet been reported, these findings, in combination with our data, raises the possibility that different Smad complexes will target different sequences to differentially affect distinct subsets of genes. In the FAST1 context, FAST1 may be making the sequence specific

DNA interaction, tethering the Smad complex to the promoter, whereas in p3TP-Lux and PAI-1, Smad4 appears to be the specific DNA-binding protein. This level of complexity may provide the diversity necessary for the regulation of a broad set of TGF-β responsive genes.

Detailed mutagenesis and methylation interference analysis of the responsive region identified a bipartite sequence as the Smad-binding site. The only apparent similarity of this binding site to that previously identified for the FAST-1/Smad2 complex is its bipartite nature. Mutation of a small, 6-nt region, was sufficient to eliminate Smad3/Smad4 binding to this probe. Functional assays with mutant reporters which lack AP1 or Smad-binding sites, revealed that the AP1 sites are required for TGF-β and Smad3/Smad4-dependent transcriptional activation. In contrast, the Smad-binding site we defined in p3TP-Lux was dispensable for both TGF-β and Smad3/Smad4-dependent transcriptional activation. The apparent dispensability of the Smad binding site within this reporter could be explained in several ways. Smad complex binding may be having effects which are not assayed in these transient transfection experiments. If, for example, Smad binding plays a role in the recruitment of other transcription factors to adjacent sites (e.g. AP1), or in re-arrangement of chromosome structure to provide accessibility of other transcription factors to their binding sites, an effect in the transient transfection assay may be difficult to observe. The transient nature of Smad DNA-binding would be consistent with this type of role in transcriptional activation. Alternatively, in the context of our artificial promoter constructs, Smad binding may not be required, but in other promoter contexts, Smad binding may be essential. Our demonstration that an endogenous promoter, PAI-1, contains a Smad3/Smad4 binding site provides an opportunity to dissect *in vivo* functions of the Smad3/Smad4 binding site and should provide insight into these important questions.

Although the functional consequences of Smad binding remains uncertain, we have clearly demonstrated that Smad3/Smad4 co-overexpression can activate transcription through AP1 binding sites. This raises the possibility that the Smads have at least two separable functions. One is a direct effect through its sequence specific DNA binding. The second is a potentially more indirect

effect to activate AP1-mediated transcription, and may explain the widely observed phenomenon that TGF-β can activate transcription through AP1 binding sites.

Finally, we present evidence that Smad4 is a DNA binding protein. Obviously, more work is required to determine if this function of Smad4 is at the root of its tumor suppressor activity. For example, defining the domain of Smad4 required for its DNA-binding ability and determining if known Smad4 mutations interfere with this activity. However, our results do raise the exciting possibility that Smad4 functions by targeting distinct heteromeric Smad complexes to various promoters to affect their transcription. Identifying these Smad4 binding site targets could greatly enhance our understanding of the function of this protein, and of its role as a tumor suppressor.

Figure Legends

Figure 1. Smad overexpression regulates transcription. (A) Diagram of the AP1 containing luciferase reporters. (B) p3TP-Lux is activated both by TGF-β and by Smad3/Smad4 co-transfection. Briefly, $2x10^6$ Mv1Lu cells were co-transfected with 3 ug of p3TP-Lux, 0.5 ug pCMV β-gal and either 2 ug pCGN vector (M) or 1 ug of the indicated Smad and 1 ug pCGN (2, 3, 4) or 1 ug Smad4 and 1ug of either Smad2 or Smad3 (2/4, 3/4). 12 hours after transfection 100 pM TGF-β was added and TGF-β induced luciferase activity was assayed 20-24 hours later. (C) The 4X WT reporter is activated by TGF-β and Smad3/Smad4 co-expression like p3TP-Lux. Luciferase assays were performed as in (B) with co-transfection of 3 ug of the minimal TATA-INR reporter construct or the 4X WT reporter construct with vector alone (M) or the combination of Smad3 and Smad4 (3/4). Luciferase assays were performed in duplicate at least three times and are standardized against β-gal expression as an internal control.

Figure 2. Co-overexpression of Smad3 and Smad 4 in COS cells changes the 2.0 probe binding profile. (A) Gel shifts were performed using the 2.0 probe derived from p3TP-Lux and extracts derived from COS cells transiently transfected with either 7 ug of pCGN (M, Lane 1), 2 ug of Smad2 (2, Lane 2) or Smad3 (3, Lane 3), 5 ug of Smad4 (4, Lane 4), or 2 ug of Smad2, 3, or 3MT in combination with 5 ug of Smad4 (2/4, Lane 5; 3/4, Lane 6; 3MT/4, Lane 7). DNA amount was kept constant at 7 ug with added pCGN vector. An HA/Flag western blot was performed to confirm expression of these proteins (Inset panel). (B) Smad 3 and Smad4 participate in a binding complex. Supershifts using antibodies described in the methods were performed on COS cells transiently transfected as in (A) with pCGN vector alone (Mock), Smad4-Flag (Flag 4), HA-Smad3/Smad4-Flag (HA 3/Flag 4). Fos: Pan Fos antibody; Fg: Flag epitope antibody; HA: anti-HA epitope antibody.

Figure 3. Identification of the Smad DNA-binding element in the 2.0 probe. (A) Diagram of the 2.0 wild type probe and mutants. Lower case letters indicate mutations introduced into the wild-type nucleotide sequence. (B) Identification of the Smad DNA-binding region. Gel shifts were performed using the indicated probes as diagrammed in (A) and COS cells transiently transfected as in Figure 2 with pCGN vector alone (M) or HA-Smad3/Smad4-Flag (3/4). (C) Methylation implicates a guanine residue within scanning mutant #2 as binding the Smad3/Smad4 complex. Methylation interference was performed using methylated 2.0 probe and COS cells transiently co-transfected with HA-Smad3 and Smad4-Flag as in Figure 2. The protected guanine residues are indicated by a • in the diagram in Figure 3A. (D) Both sites on the 2.0 repeat sequence are required for Smad3/Smad4 binding. Gel shifts were performed using the indicated probes as diagrammed in (A) and COS cells transiently transfected with pCGN vector alone (M) or HA-Smad3/Smad4-Flag (3/4).

Figure 4. Purified GST-Smad4, but not GST-Smad3 directly binds DNA. GST-fusion protein construction is described in *Materials and Methods*. Gel shifts were performed with

2 ul (Lanes 1 and 3) or 1 ul (Lanes 2 and 4-10) of either GST-Smad3 (Lanes 1 and 2) or GST-Smad4 (Lanes 3-10) at a concentration of ~100 ng/ul and the indicated probes as diagrammed in Fig. 3A and the following antibodies: S4, Smad4 antibody; Pre, preimmune serum.

Figure 5. Induction of a Smad3/Smad4 DNA-binding complex in Mv1Lu cells by TGF-β. (A) Gel shift analysis with the 2.0 probe with lysates from a time course of TGF-β treatment of Mv1Lu cells. (B) The inducible complex is sensitive to phosphatase treatment. Mv1Lu lysates from control (Lanes 1, 3 and 5) or TGF-β treated (Lanes 2, 4 and 6) cells were treated with PAP+CIAP (Lanes 1-4) or phosphatase inhibitors (Lanes 3-6) as described in *Materials and Methods*. (C) Supershift analysis on the induced complex in Mv1Lu cells. Gel shifts were performed with the 2.0 probe as diagrammed in Fig. 3A and the indicated antibodies: Fos, Pan Fos antibody; S4, Smad4 antibody; Pre, preimmune serum. Bottom panel, Western showing the specificity of the Smad4 antibody. Left panel is an HA antibody Western showing expression of Smad1-4; right panel is a duplicate Western blot with Smad4 antibody. The Smad4 antibody is also specific by immunoprecipitation analysis (data not shown). (D) Analysis of the 2.0 probe mutants with TGF-β treated Mv1Lu cell lysates. Lysates were prepared from control (odd numbered lanes) or TGF-β treated cells (even numbered lanes) and probes indicated are as shown in Fig. 3A.

Figure 6. Functional analysis of the 2.0 Probe mutants. Mv1Lu cells were transfected with 2 ug vector DNA (M) or with 1 ug HA-Smad3 and Smad4-Flag DNA (3/4) along with 3 ug of the indicated reporter and 0.5 ug pCMV β-gal. Luciferase assays were performed as described in Figure 1.

Figure 7. Endogenous PAI-1 Promoter Contains a Smad3/Smad4 Binding Site. (A) Schematic diagram of the PAI-1 promoter. The black boxes represent potential AP1 binding sites. The shaded region (-792 to -644) represents the probe used in gel shift assays. (B) Gel shift analysis using the probe from (A) was performed with COS lysates from cells transfected as in Fig. 2 with pCGN vector alone (left lane) or HA-Smad3/Smad4-Flag (right lane).

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Figure 1 A

A

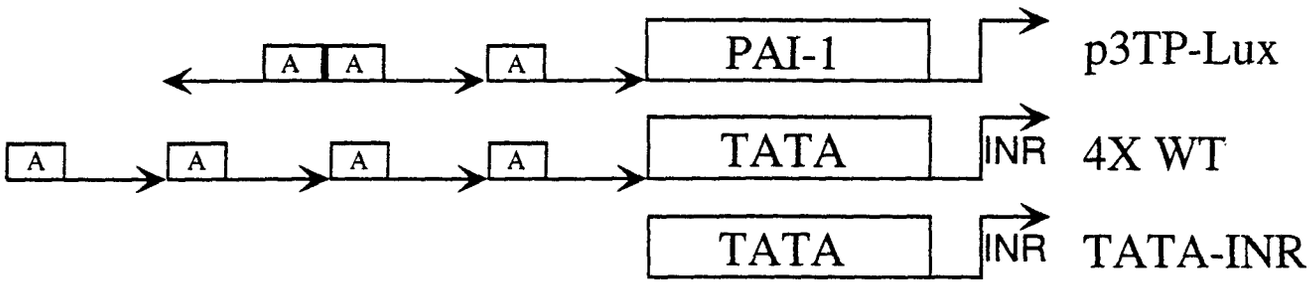
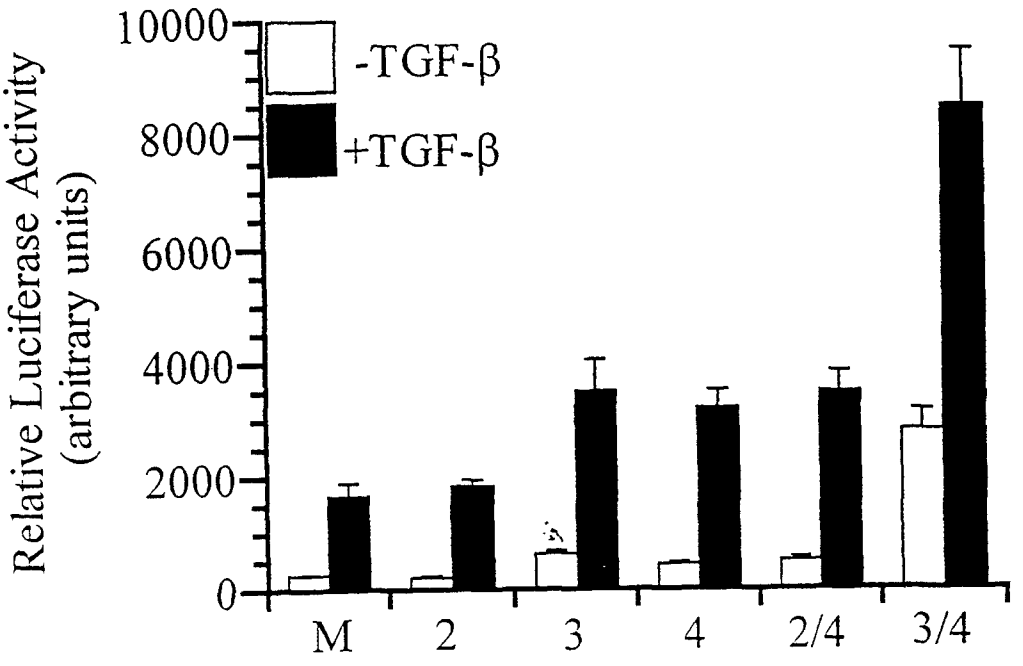


Figure 1 B & C

B



C

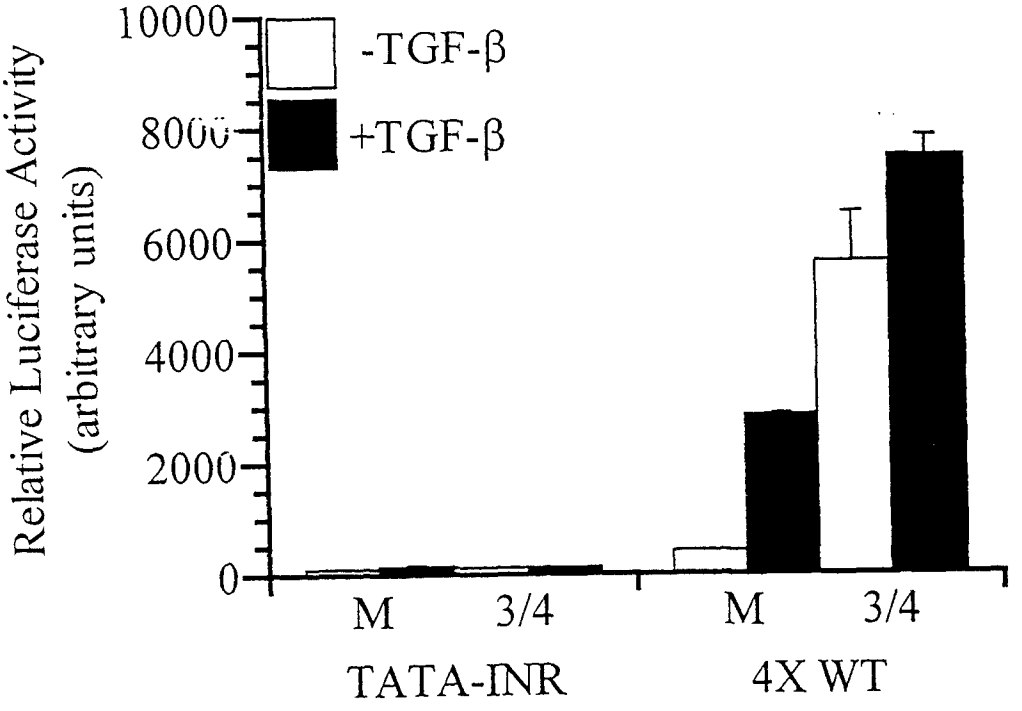


Figure 2A

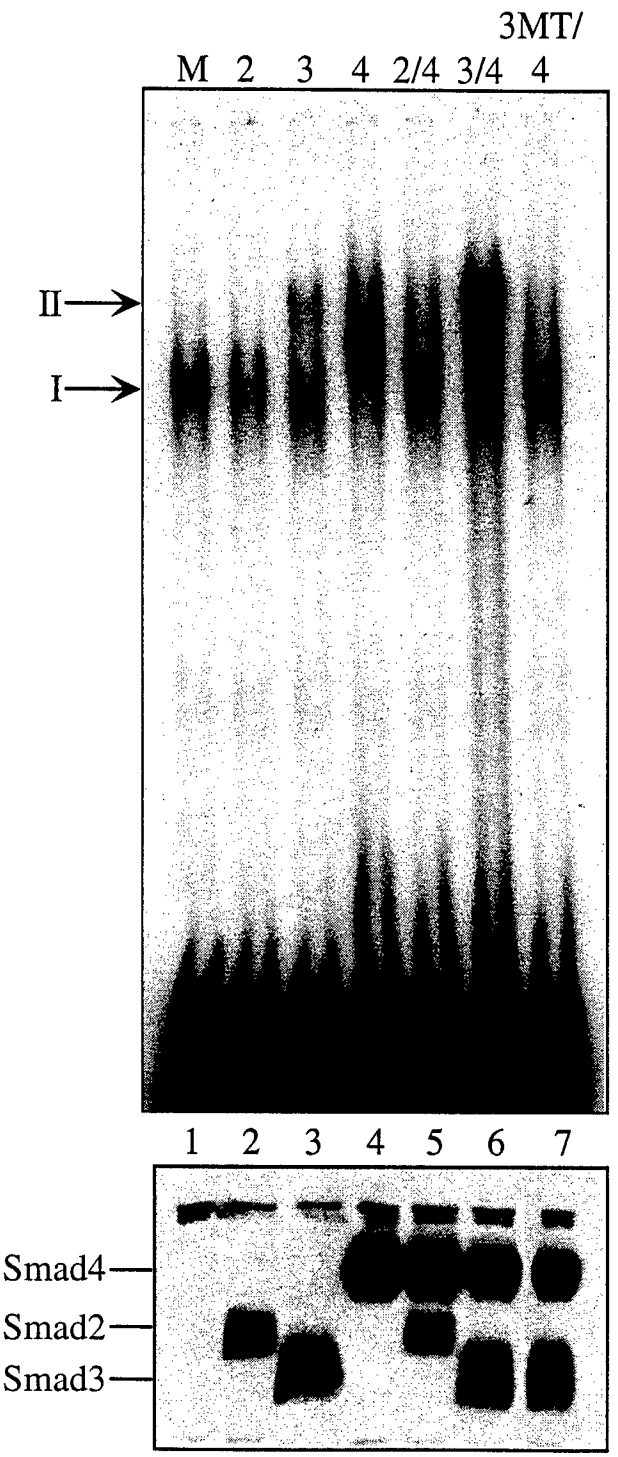


Figure 2B

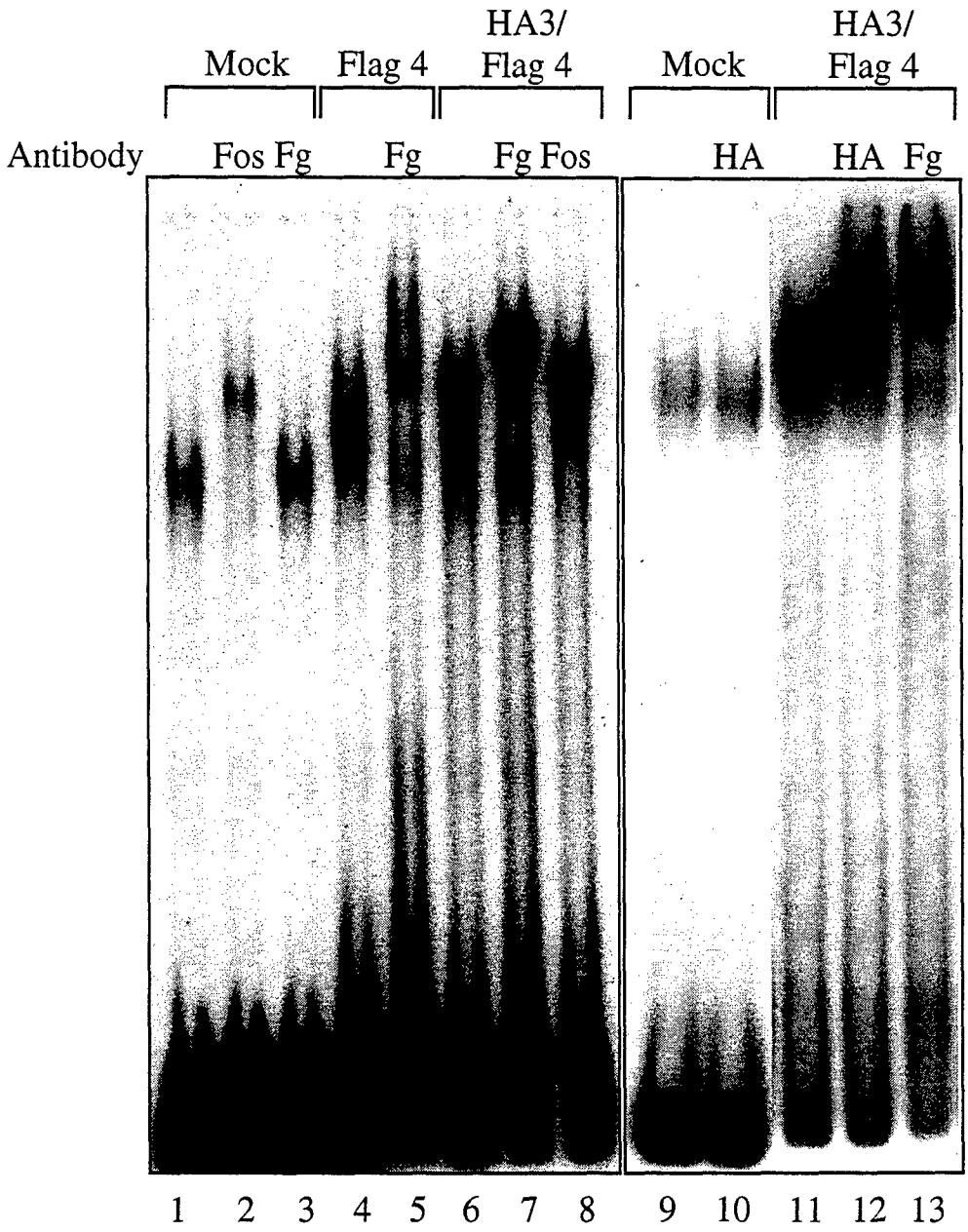

Mock
Flag 4
HA3/
Flag 4
Mock
HA3/
Flag 4
Antibody
Fos Fg
Fg
Fg Fos
HA
HA Fg
1 2 3 4 5 6 7 8 9 10 11 12 13

Figure 3 A

A

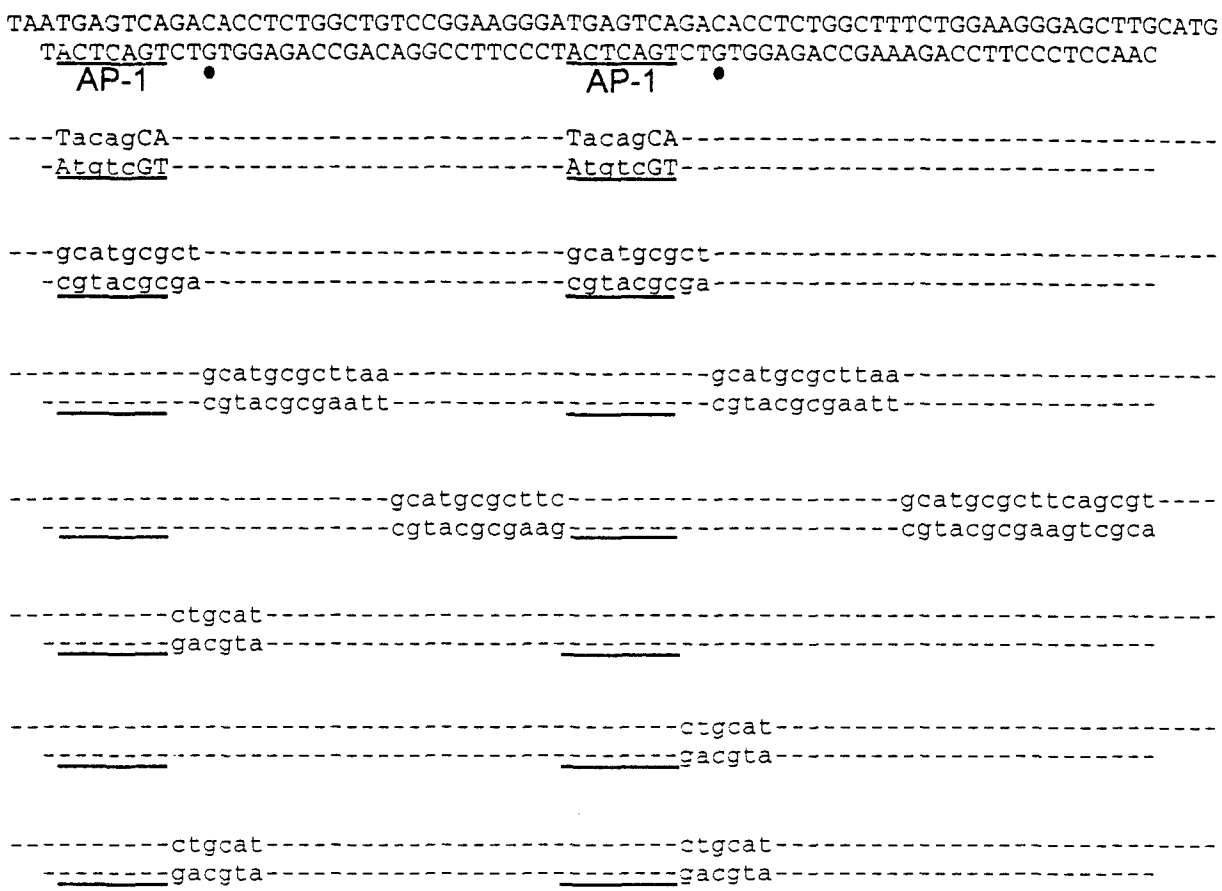

TAATGAGTCAGACACCTCTGGCTGTCCGGAAGGGATGAGTCAGACACCTCTGGCTTTCTGGAAGGGAGCTTGCATG
TACTCAGTCTGTGGAGACCGACAGGCCTTCCCTACTCAGTCTGTGGAGACCGAAAGACCTTCCCTCCAAC
AP-1
AP-1
---TacagCA----------TacagCA----------
-AtgtcGT----------AtgtcGT----------
---gcatgcgct----------gcatgcgct----------
-cgtacgcga----------cgtacgcga----------
----------gcatgcgcttaa----------gcatgcgcttaa----------
---------cgtacgcgaatt----------cgtacgcgaatt----------
----------gcatgcgcttc----------gcatgcgcttcagcgt----
-------cgtacgcgaag-------cgtacgcgaagtcgca
----------ctgcat----------
-------gacgta----------
----------ctgcat----------
-------gacgta----------
----------ctgcat----------ctgcat----------
-------gacgta----------gacgta----------

Figure 3B

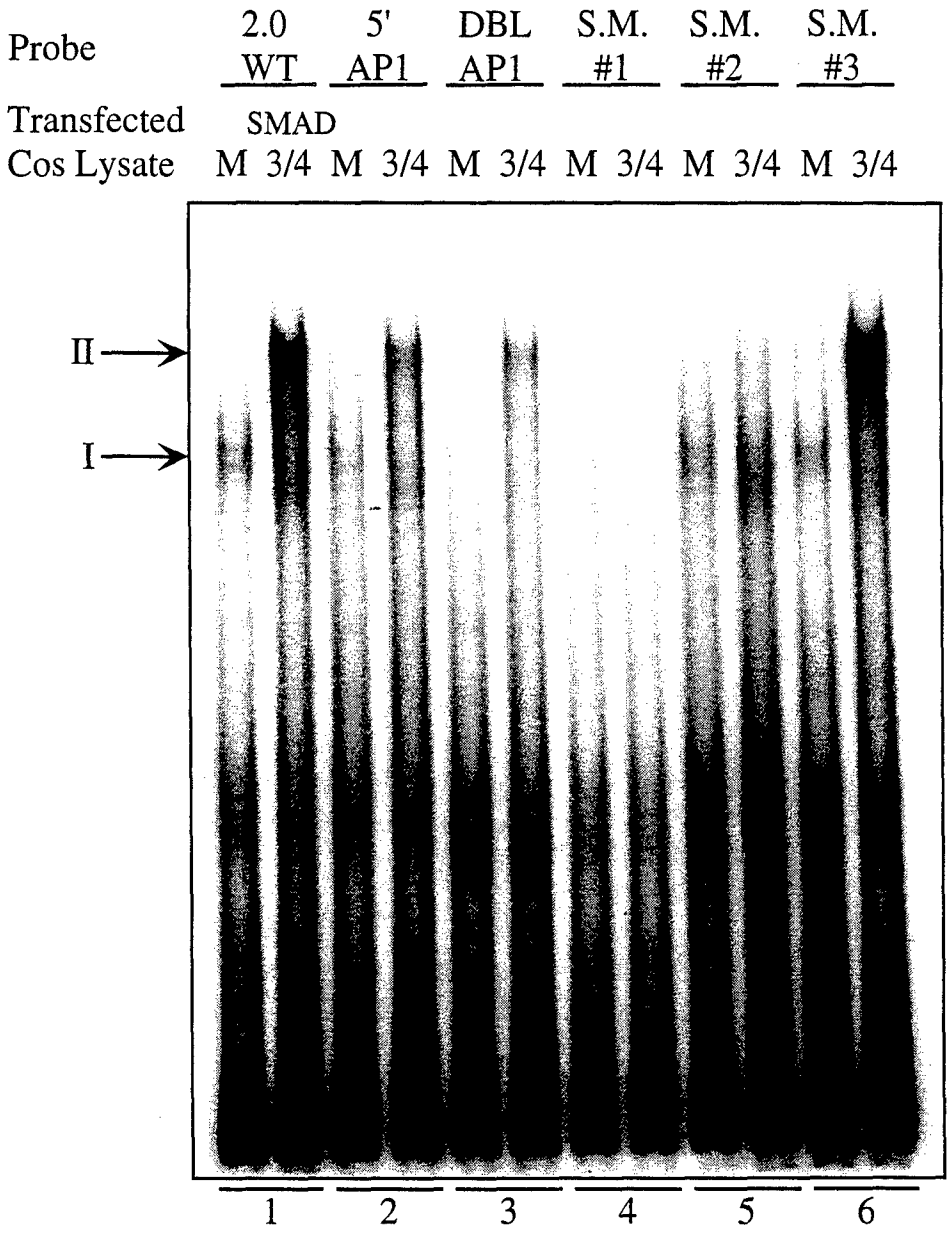

Probe
2.0 WT
5' AP1
DBL AP1
S.M. #1
S.M. #2
S.M. #3
Transfected Cos Lysate
SMAD
M 3/4 M 3/4 M 3/4 M 3/4 M 3/4 M 3/4
II
I
1
2
3
4
5
6

Figure 3C

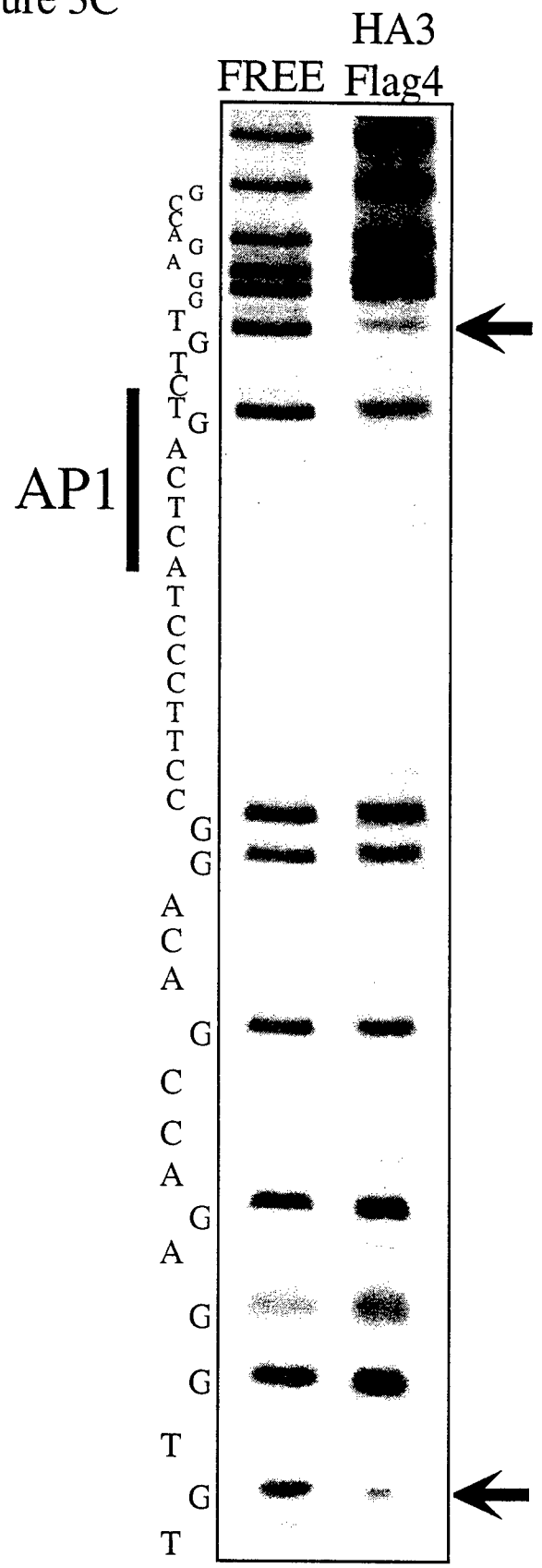

HA3
FREE
Flag4
AP1
G
C
C
A
A
G
G
G
T
G
T
C
T
G
A
C
T
C
A
T
C
C
C
T
T
C
C
G
G
A
C
A
G
C
C
A
G
A
G
G
T
G
T

Figure 3D

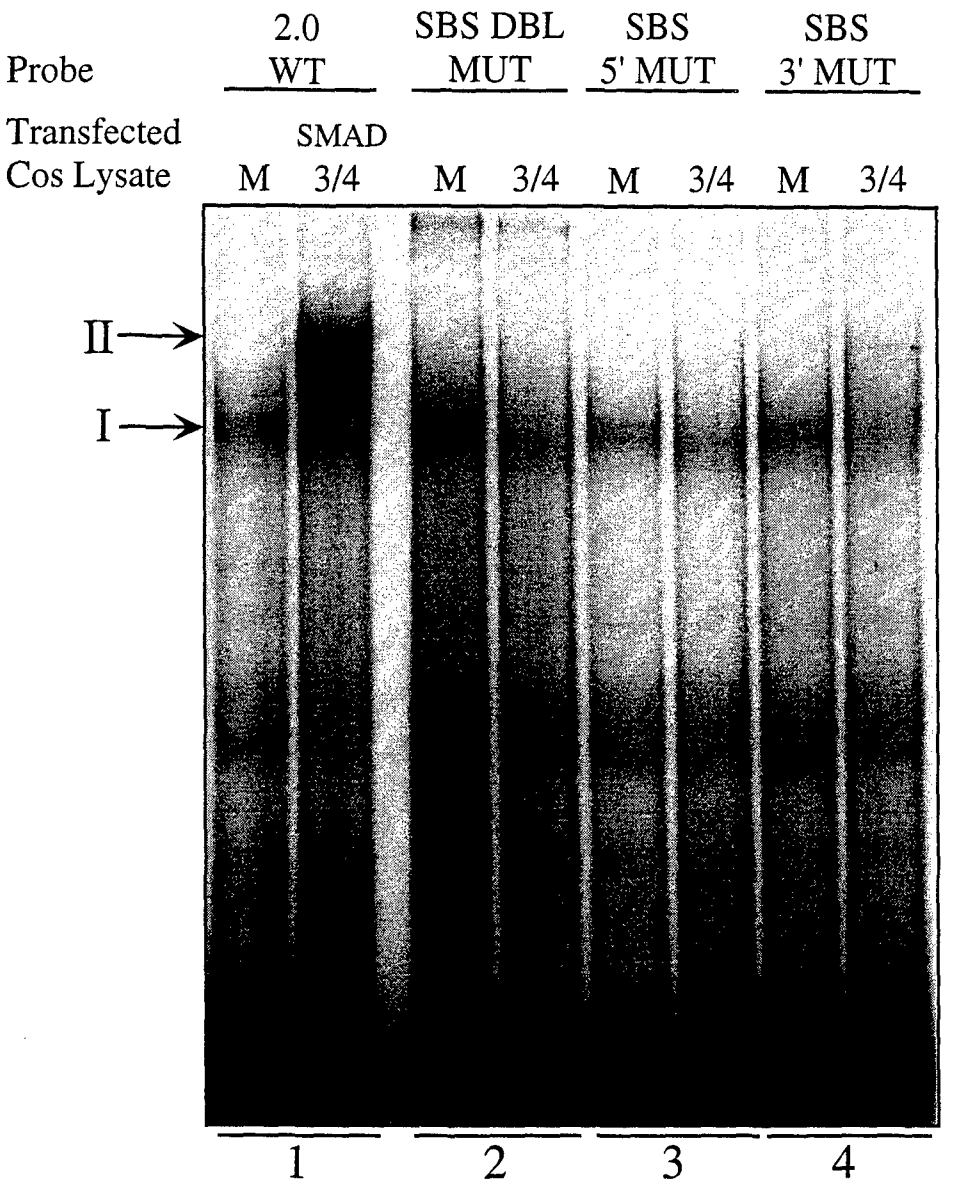

Probe
2.0 WT
SBS DBL MUT
SBS 5' MUT
SBS 3' MUT
Transfected Cos Lysate
M
SMAD 3/4
M
3/4
M
3/4
M
3/4
II
I
1
2
3
4

Figure 4

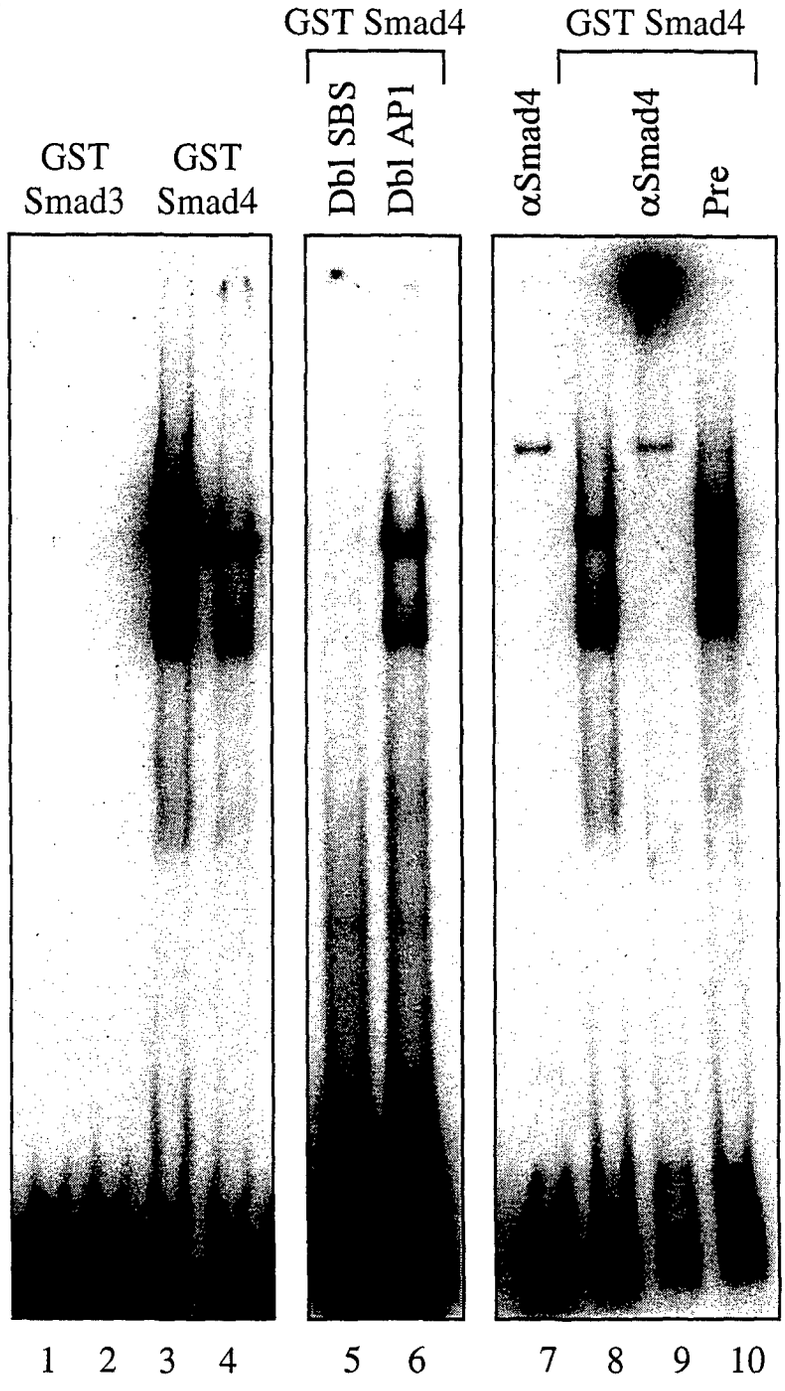

GST Smad4
GST Smad4
GST Smad3
GST Smad4
Dbl SBS
Dbl AP1
αSmad4
αSmad4
Pre
1 2 3 4 5 6 7 8 9 10

Figure 5A

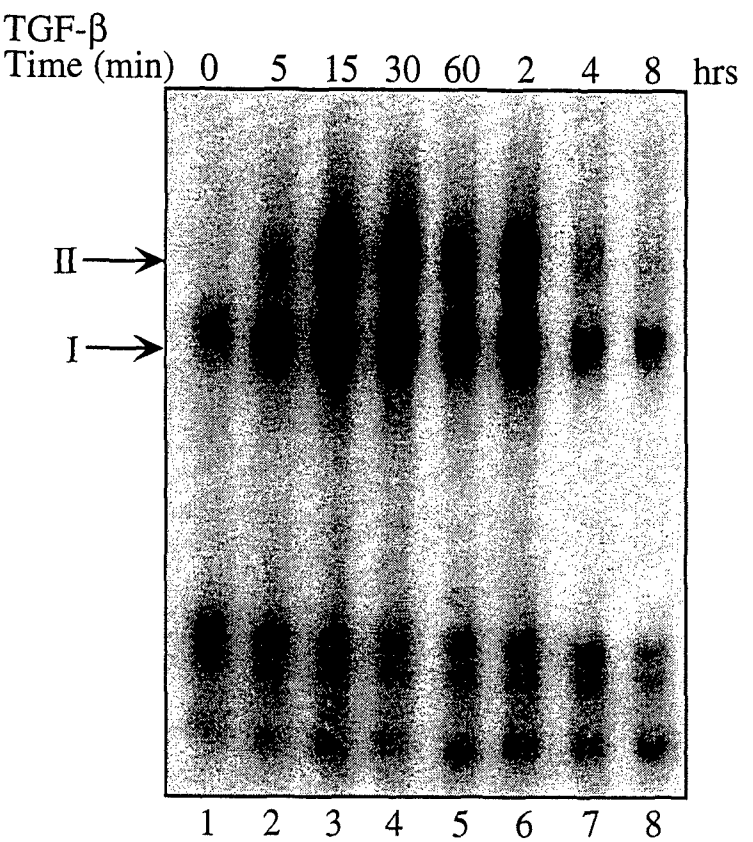


Figure 5B

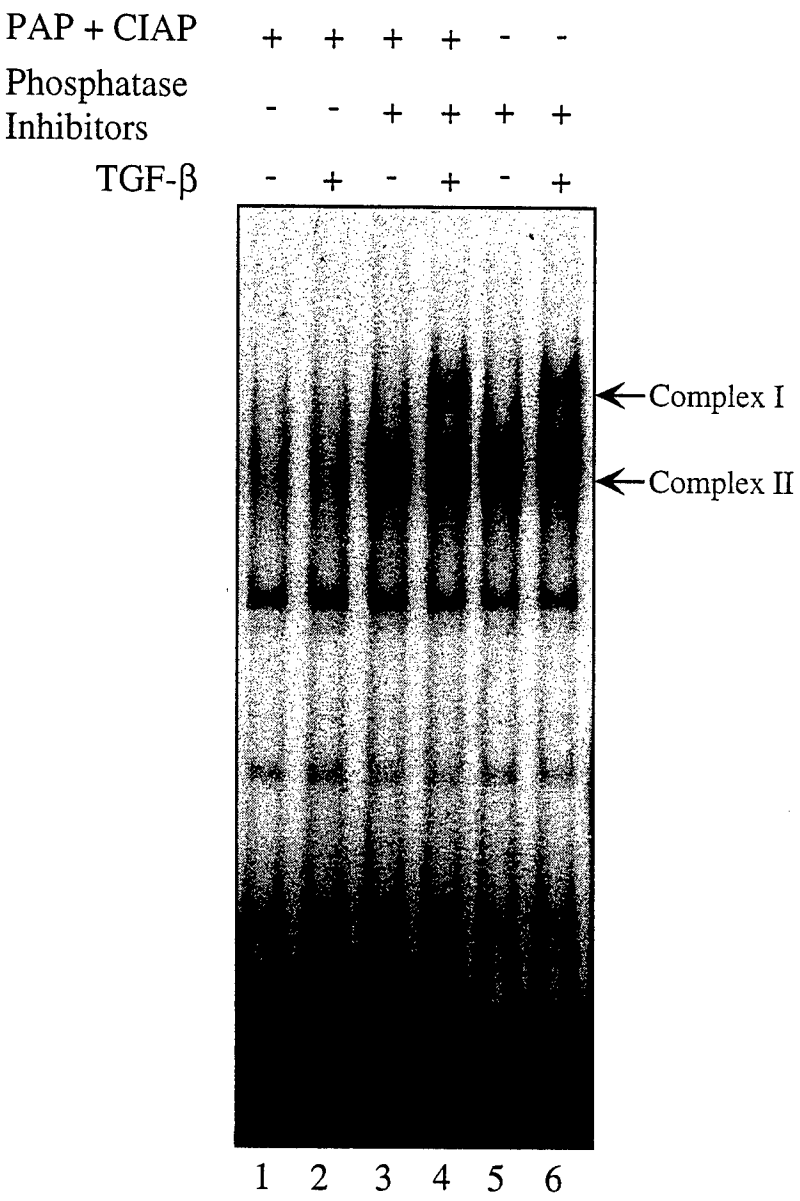

PAP + CIAP + + + + - -
Phosphatase Inhibitors - - + + + +
TGF-β - + - + - +
Complex I
Complex II
1 2 3 4 5 6

Figure 5C

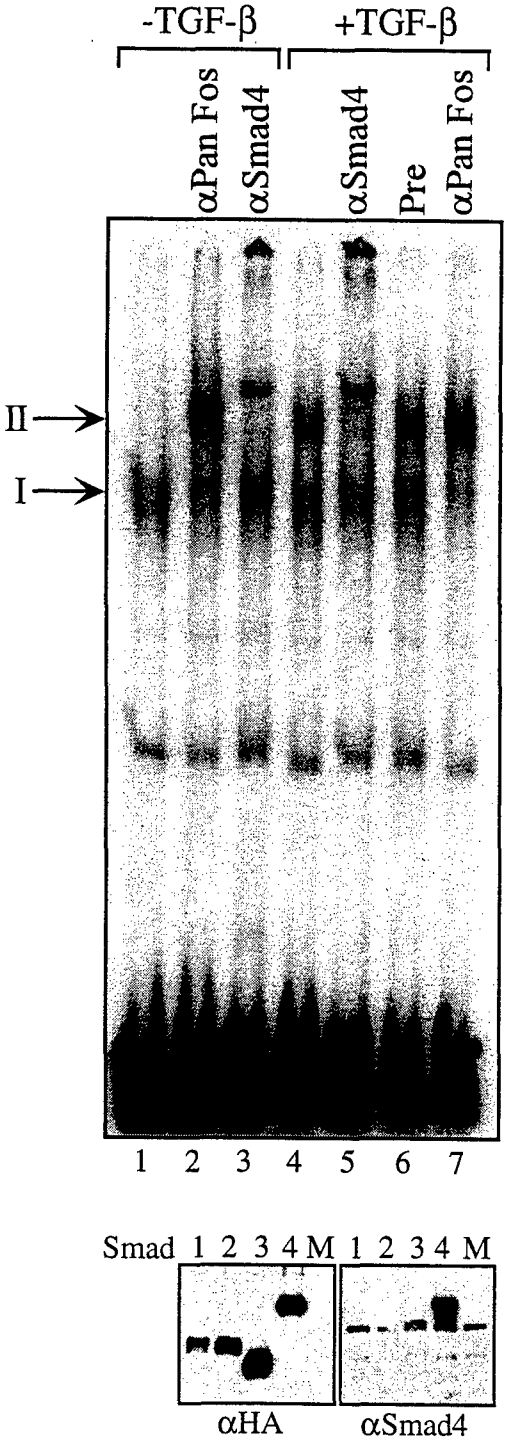

-TGF-β
+TGF-β
αPan Fos
αSmad4
αSmad4
Pre
αPan Fos
II
I
1 2 3 4 5 6 7
Smad 1 2 3 4 M 1 2 3 4 M
αHA
αSmad4

Figure 5D

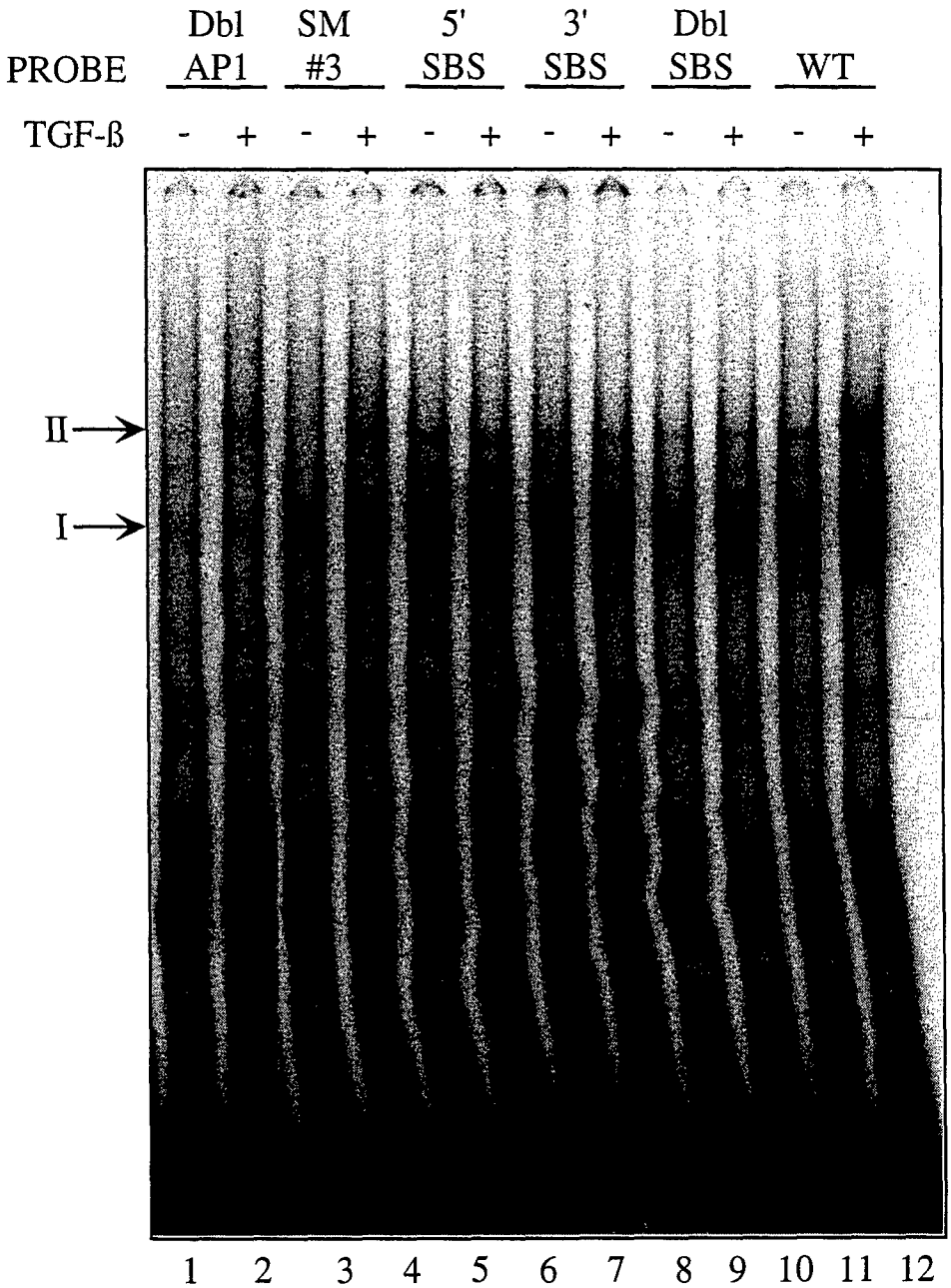

PROBE
Dbl AP1
SM #3
5' SBS
3' SBS
Dbl SBS
WT
TGF-ß
- + - + - + - + - + - +
II
I
1 2 3 4 5 6 7 8 9 10 11 12

Figure 6

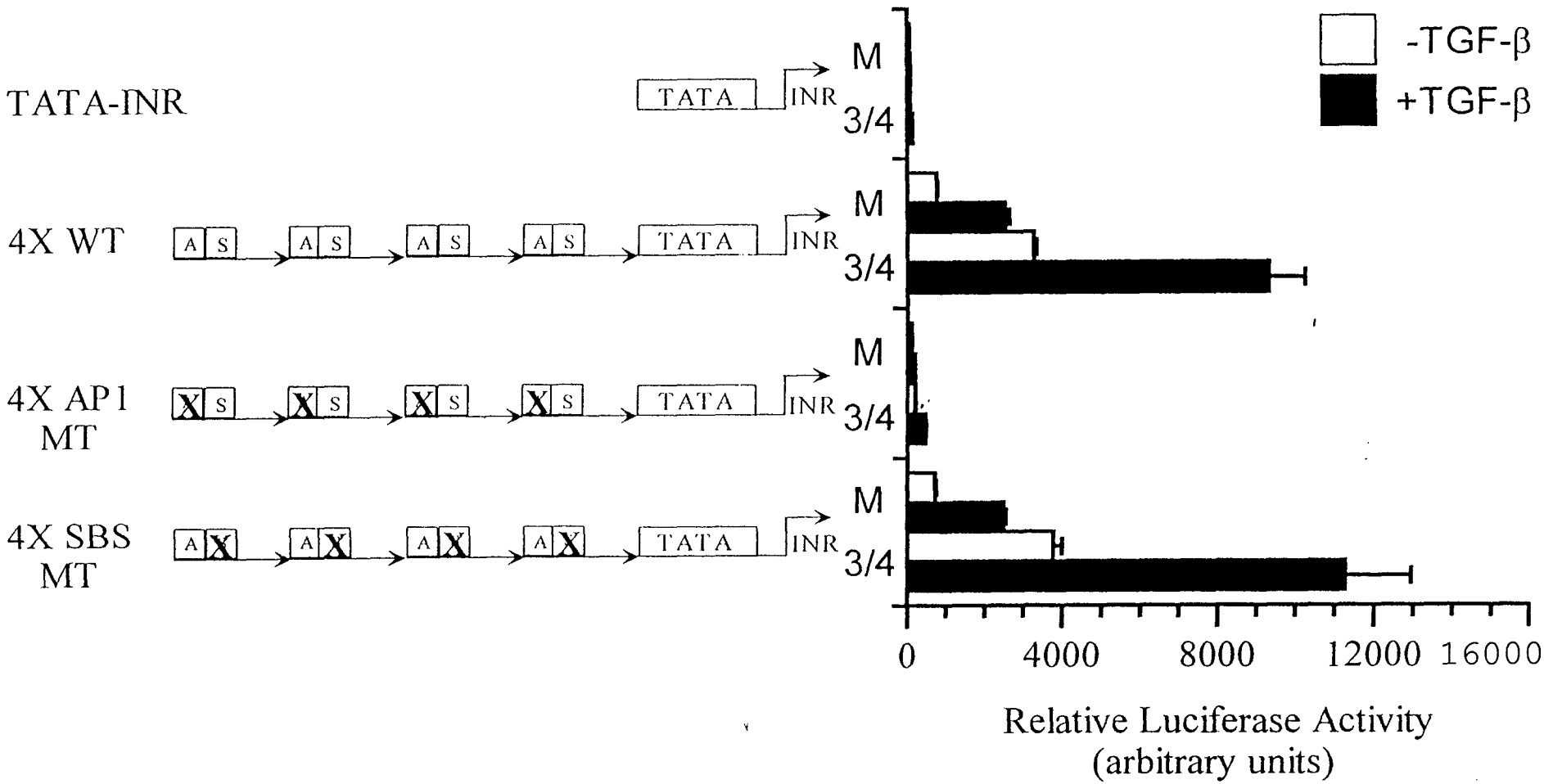
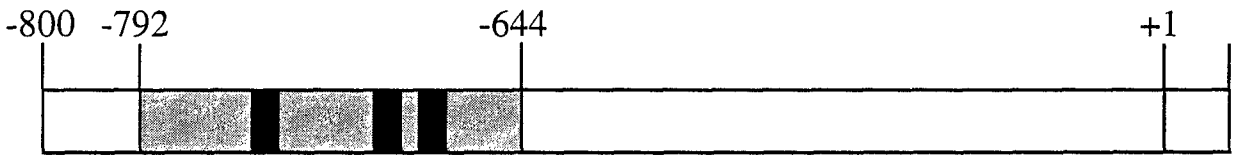

TATA-INR
TATA
INR
M
3/4
-TGF-β
+TGF-β
4X WT
A S
TATA
INR
4X AP1 MT
X S
4X SBS MT
A X
0
4000
8000
12000
16000
Relative Luciferase Activity
(arbitrary units)

Figure 7

A



PAI-1 promoter region